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Optimization of crude enzyme preparation methods for analysis of glutamine synthetase activity in phytoplankton and field samples

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Abstract
Glutamine synthetase (GS) is an important enzyme involved in nitrogen assimilation and metabolism in marine phytoplankton. However, little work has been done in situ due to the limitation of crude enzyme preparation methods. In this study, three enzyme preparation methods, high-speed centrifugation (HC, <10 000 g), ultracentrifugation (UC, 70 000 g), and ultrafiltration (UF) with 100 kμ molecular weight cutoff, were compared using two diatom species (*Asterionellopsis glacialis* and *Thalassiosira weissflogii*), and two dinoflagellate species (*Alexandrium catenella* and *Prorocentrum donghaiense*) as experimental materials together with field samples collected from Xiamen Harbor, China. The results showed that HC is the best method to prepare crude enzymes for glutamine synthetase activity (GSA) in diatom species and diatom-dominant samples, while UF is the best method to extract GS from dinoflagellate species and dinoflagellate-dominant samples. For the HC method, the optimal centrifugal speed and time were 10 000 g and 35 min, respectively, and under these conditions, the highest GSA was obtained in all samples. This study indicates that both methods (HC and UF) overcome the limitation of centrifugal speed and could be applied to in situ GSA analysis, especially at sea.

Key words: glutamine synthetase, ultracentrifugation, centrifugation, ultrafiltration, phytoplankton

1 Introduction

Nitrate and ammonium are the major forms of inorganic nitrogen that usually limit phytoplankton productivity in the sea, especially during the periods of surface stratification (Codispoti, 1983; McCarthy and Carpenter, 1983; Ryther and Dunstan, 1971). In the past few decades, many efforts have been devoted to marine nitrogen cycling, the metabolic pathway and regulating mechanism of nitrogen in phytoplankton (Schaefer et al., 2000, 1999; Smith et al., 1992). It is known that the utilization of nitrogen in phytoplankton involves various nutrient assimilation enzymes inside the cells (Dortch and Postel, 1989), e. g. nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS). Among these enzymes, GS plays important roles in regulating nitrogen metabolism not only as the center for both nitrate and ammonium assimilation, but also as a key enzyme linking carbon to nitrogen metabolism for it incorporates inorganic nitrogen into organic nitrogen via GS/glutamate synthase (GOGAT). Many organisms regulate GS activity (GSA) in response to changes in the cellular C∶N ratio (Maurin and Gal, 1997; Deborah and Randall, 1996; Humanes et al., 1995; Sivasankar and Oaks, 1995; Reyes and Florencio, 1994).

Since Bressler and Ahmed (1984) first reported the wide distribution of GS in a variety of marine phytoplankton, GS has been studied extensively in many phytoplankton and algal species (Ei Alaoui et al., 2003, 2001; García-Fernández et al., 1997; Robertson and Alberte, 1996; Humanes et al., 1995; García-Fernández et al., 1994). However, almost all these studies were conducted in the laboratory, little work has been done in situ samples due to the limitation of GS extraction method. Usually, GS was extracted from algal or phytoplankton samples using centrifuge with high speed. Bressler and Ahmed (1984) extracted GS using 20 400 g centrifugal speed in their study of GSA of algae. Robertson and Alberte (1996) established a two-step extraction method: firstly extracting GS using 10 000 g for 10 min at 4 °C, then using 43 000 g for 15 min at 4 °C.

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García-Fernández et al. (1997, 1994) and Humanes et al. (1995) used the same method to prepare GS crude extract for GSA analysis: the crude extract was centrifuged at 70 000 g for 50 min, then streptomycin sulphate was added to remove pigments between the intermission of two centrifugations. After 15 min at 4 °C with continuous stirring, the supernatant was centrifuged again at 70 000 g for 50 min. Meanwhile some other investigators tried to use low centrifugal speed to prepare GS crude extract (Ei Alaoui et al., 2003, 2001; Maurin and Gal, 1997). Although there have been lots of work on GSA analysis in phytoplankton, no universal GS preparation method has been proposed, the centrifugal speed and time varied greatly among different studies, thus resulting in uncertainty and incompatibility of the data. Many studies used high centrifugal speed (more than 20 000 g), which is impractical for GSA analysis in situ, especially at sea. It is thus essential and urgent to establish a universal GS preparation method, especially for in situ GSA analysis.

In this study, we compared three GS preparation methods, high-speed centrifugation (HC, <10 000 g), ultracentrifugation (UC, 70 000 g), and ultrafiltration (UF) with 100 kμ molecular weight cutoff using two diatom and two dinoflagellate species as experimental materials together with field samples. The goal of this study is to establish and optimize crude GS preparation method for phytoplankton species and in situ GSA study.

2 Materials and methods

2.1 *Culture conditions*

Asterionellopsis glacialis (Castracane) Round and *Thalassiosira weissflogii* (Grunow) Fryxell and Hasle were isolated from the Taiwan Strait. *Alexandrium catenella* (Whedon and Kofoid) Balech and *Prorocentrum donghaiense* Lu were isolated from the East China Sea. The four microalgal species were grown in natural seawater supplemented with f/2 medium (Guillard and Ryther, 1962) for diatoms and K medium (Keller, 1987) for dinoflagellates at a regime of 20 °C temperature, 80 μE·m^{-2}·s^{-1} light intensity and 12h∶12 h light∶dark cycle. When the cells to the middle exponential phase, cells were harvested for GSA analysis. The field samples for GSA analysis were collected from the surface seawater of Xiamen Harbor, China (about 24.365°N, 118.125°E).

2.2 *Comparing GS preparation methods*

Both cell pellets and the field samples were filtered on to the nuclearpore membranes (0.45 μm) and broken immediately in an ice-water slurry of extraction buffer containing 50 mmol/dm^3 Tris-HCl, pH 7.5, 2 mmol/dm^3 dithiothreitol (DTT), 1 mmol/dm^3 ethylenediaminetetraacetic acid (EDTA), 2.5 mmol/dm^3 $MgCl_2$ (García-Fernández et al., 1997; Humanes et al., 1995) with the help of sonicator. The homogenate was then divided into three parts evenly. The first part was treated using the UC method with the centrifugal speed of 70 000 g for 50 min, then a solution of 100 mmol/dm^3 streptomycin sulphate (pH 7.0) was added to the supernatant (0.1 ml/ml). After stirring for 15 min continuously, the mixture was centrifuged at 70 000 g for 50 min again and the supernatant was collected for GSA analysis. The molecular analysis of GS showed that the molecular weight of GS of alga were around 70 kμ, lower than 100 kμ (García-Fernández et al., 1997; Robertson and Alberte, 1996). As a result, the second part was treated by the ultrafiltration (UF) method with 100 kD molecular weight cutoff (Microcon, YM-100). The filtrate with molecular weight less than 100 kμ was collected and 100 mmol/dm^3 streptomycin was added to remove pigments, and after stirring continuously for 15 min, the filtrate was centrifuged again, and the filtrate with molecular weight less than 100 kμ was collected for GSA analysis. The third part was treated using the high-speed centrifugation (HC, <10 000 g) method with the centrifugal speed of 8 000 g for 30 min, and subsequently a solution of 100 mmol/dm^3 streptomycin sulphate was added to the supernatant (0.1 ml/ml). After stirring continuously for 15 min, the supernatant was centrifuged at 8 000 g for 30 min and the supernatant was collected for GSA analysis. All above operations were conducted at 4 °C.

2.3 *Analysis of enzyme activities*

GSA was analyzed using transferase assay according to Shapiro and Stadtman (1970) by measuring the γ-glutamylhydroxamate produced. Briefly, to the first tube, 100 μl of enzyme crude extract together with the reaction buffer containing 960 μl 1.0 mol/dm^3 imidazole-HCl buffer, 600 μl 0.1 mol/dm^3 glutamine (pH 7.3), 60 μl 0.01 mol/dm^3 $MnCl_2$, 80 μl 0.01 mol/dm^3 ADP (pH 7.3), 40 μl 1.0 mol/dm^3 K-arsenate and 60 μl 2.0 mol/dm^3 hydroxylamine were added. To the second tube, the enzyme crude extract and the reaction buffer were added as a time zero con-

trol. To the third tube, only reaction buffer was added as a reagent blank. The reactions were conducted at 37 °C for 30 min and terminated by addition of a stop solution (2.0 ml of a mixture of 4.0 ml 10% $FeCl_3$, 1.0 ml 24% trichloroacetic acid, 0.5 ml 6 mol/dm^3 HCl and 6.5 ml Milli-Q water). In the case of the second tube, the reaction was terminated as soon as it started by addition of the stop solution. After stopping the reaction, the absorbance was measured at 540 nm on a Cary-100Bio UV-visible spectrophotometer.

GS biosynthetic activity was estimated according to Bressler and Ahmed (1984) by determining the amount of inorganic phosphate. Briefly, to the first tube, 100 μl of enzyme crude extract together with a reaction buffer containing 123 μl 1.0 mol/dm^3 imidazole-HCl buffer (pH 7.8), 100 μl 0.01 mol/dm^3 NH_4Cl, 20 μL 0.06 mol/dm^3 adenosine-triphosphate (ATP, pH 7.8), 17 μl 1.67 mol/dm^3 $MgCl_2$, 100 μl 1.0 mol/dm^3 glutamate (pH 7.8) were added. To the second tube, the enzyme crude extract and the reaction buffer were added as a time zero control. To the third tube only reaction buffer was added as a reagent blank. For the first and third tube, after an incubation period of 30 min at 25°C, the reactions were terminated by addition of a stop solution: 1.8 ml of $FeSO_4.7H_2O$ (0.8 w/v in 0.007 5 mol/dm^3 H_2SO_4). In the case of the second tube, the reaction was terminated as soon as it started by addition of the stop solution. After stopping the reaction, 0.15 ml of the color-forming reagent ammonium molydate (6.6% w/v in 3.75 mol/dm^3 H_2SO_4) was added. The reaction tubes were placed in an ice bath and immediately read at 850 nm on a Cary-100Bio UV-visible spectrophotometer.

For GSA analyses, a unit (U) was determined as 1 mol of product formed per hour per liter of seawater.

2.4 *Optimizing GS preparation method*

For optimizing the HC method of GS preparation, the gradient of centrifugal speed (4 000, 6 000, 8 000, 10 000 and 12 000 g) and centrifugal time (10, 20, 25, 30, 35 and 40 min) were examined and GS transferase activity was analyzed in *Th. weissflogii*.

3 Results

3.1 *Comparison of GS transferase activities using three GS preparation methods*

The GS transferase activities of the four cultures and field samples using the three different GS preparation methods are shown in Figs 1 and 2. GS

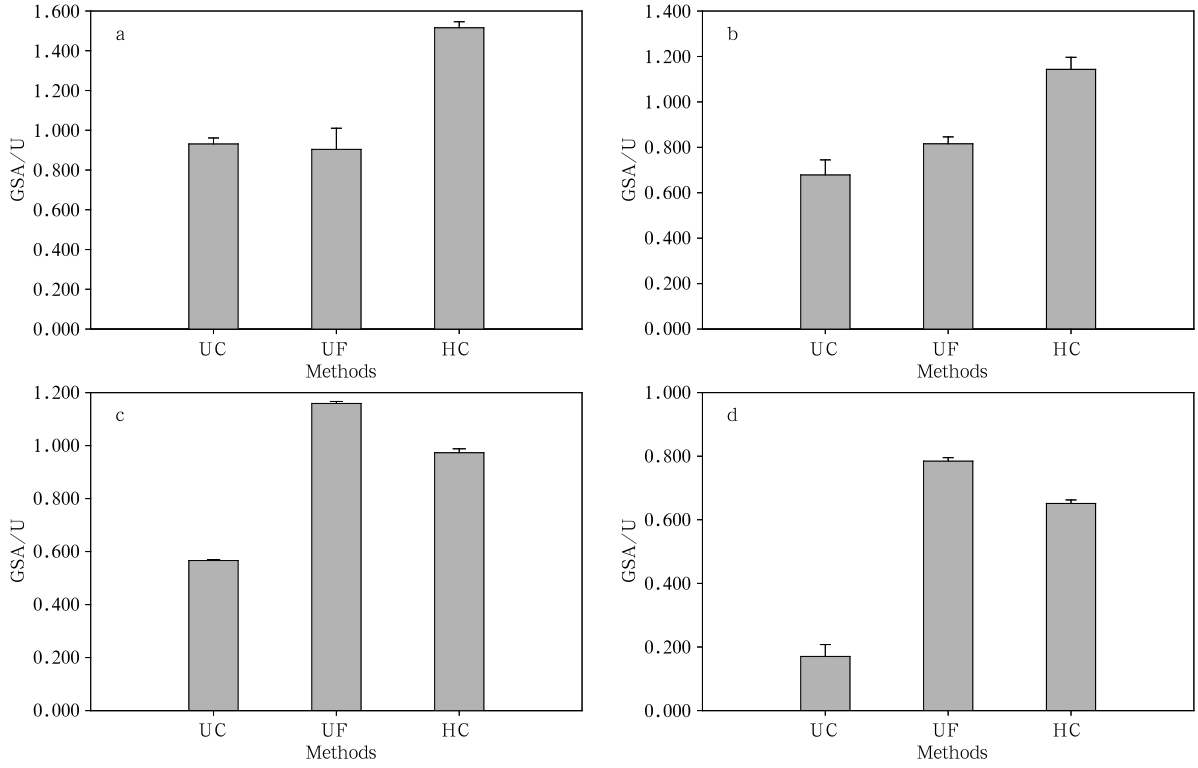

Fig.1. Comparison of GS transferase activities in diatom and dinoflagellate species using three extraction methods (UC, UF and HC). Activities are expressed as a mean value (n=3), and an error bar ($\pm SE$) is provided for each column. a– *Th. weissflogii*; b– *A. glacialis*; c– *a. catenella*; d– *P. donghaiense*.

transferase activities showed no significant difference between the two preparation methods, UC and UF, in two diatom species, however, they were much lower than that with the HC method, only about 65% of GS activity with the HC method (see Figs. 1a, b). Variations of GS transferase activities of two dinoflagellate species differed from that of the diatoms. GS transferase activities prepared with the UF method were the highest among the three methods. GS transferase activities in the extracts with the HC method were about 80% of those obtained using UF method. Meanwhile, GS transferase activities prepared with the UC method only presented about 49% (*A. catenella*) and 22% (*P. donghaiense*) of those obtained using the UF method (Figs. 1c, d).

The results of field samples collected from Xiamen Harbor, China showed good consistency with the results for the cultured diatoms (Fig. 2). GS transferase activity in the extract using the HC method was much higher than those obtained using the other two methods. GS transferase activities obtained using the UF and UC methods only represented 68% of that obtained using the HC method, indicating diatom species might dominate the phytoplankton composition in the field samples, and the HC method is an efficient method for GSA study in diatom dominant samples.

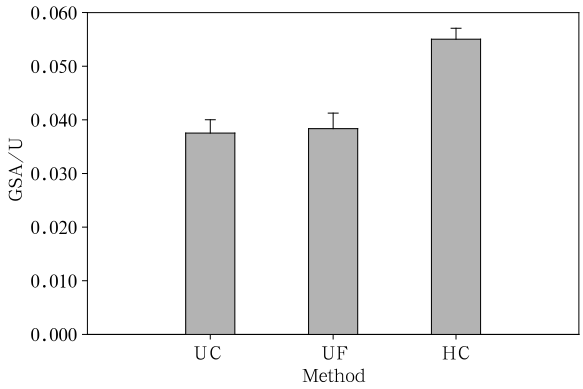

Fig.2. Comparison of GS transferase activities in field samples of Xiamen Harbor using three extraction methods (UC, UF and HC). Activities are expressed as a mean value (n=3), and an error bar ($\pm SE$) is provided for each column.

3.2 *Comparison of GS biosynthetic activities using three preparation methods*

To further prove that the HC method is an efficient method for preparing crude enzyme for GSA study in diatom species, the GS biosynthetic activities of *Th. weissflogii* in the extracts prepared with the three different methods were also compared (Fig. 3). GS biosynthetic activities showed the same trend as GS transferase activities of *Th. weissflogii*. GS biosynthetic activity in the extract prepared with the HC method was much higher than those obtained using the UC and UF methods, and no significant difference was observed between the latter two methods. GS biosynthetic activities obtained using the UC and UF methods only presented about 40% of that with the HC method.

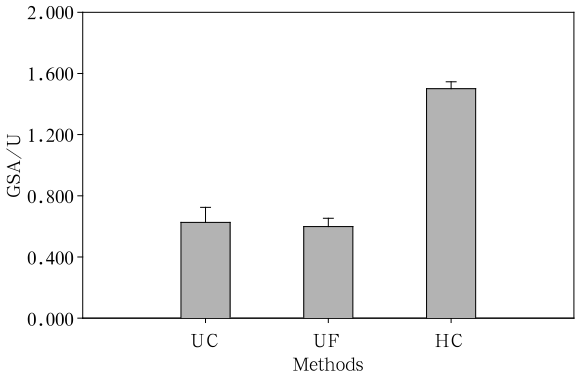

Fig.3. Comparison of GS biosynthetic activities of *Th. weissflogii* using the three preparation methods (UC, UF and HC). Activities are expressed as a mean value (n=3), and an error bar ($\pm SE$) is provided for each column.

3.3 *Optimization of the HC method*

Previous studies have reported various centrifugal speeds and times for the preparation of crude GS extracts (García-Fernández et al., 1997; Robertson and Alberte, 1996; Humanes et al., 1995; García-Fernández et al., 1994), however no study investigated the effects of these two parameters on GSA. Based on the above results and considering the importance of diatoms in marine environment, the HC method was selected to optimize the centrifugal speed and time using *Th. weissflogii* as the experimental specie.The GS transferase activities of *Th. weissflogii* crude extracts prepared with the HC method were investigated under various of centrifugal speeds and times, and the results showed that the GSA increased with the centrifugal speed in the range of 4 000 g to 10 000 g with the highest value of 0.620 U at 10 000 g (Fig. 4b), then decreased when the centrifugal speed reached 12 000 g. GSA increased with the centrifugal time in the range of 10 to 35 min at the centrifugal speed of

10 000 g and reached the highest at 35 min, then it decreased with the further increase of centrifugal time. Thus, the optimum centrifugal speed and time for GS extraction was 10 000 g and 35 min, respectively.

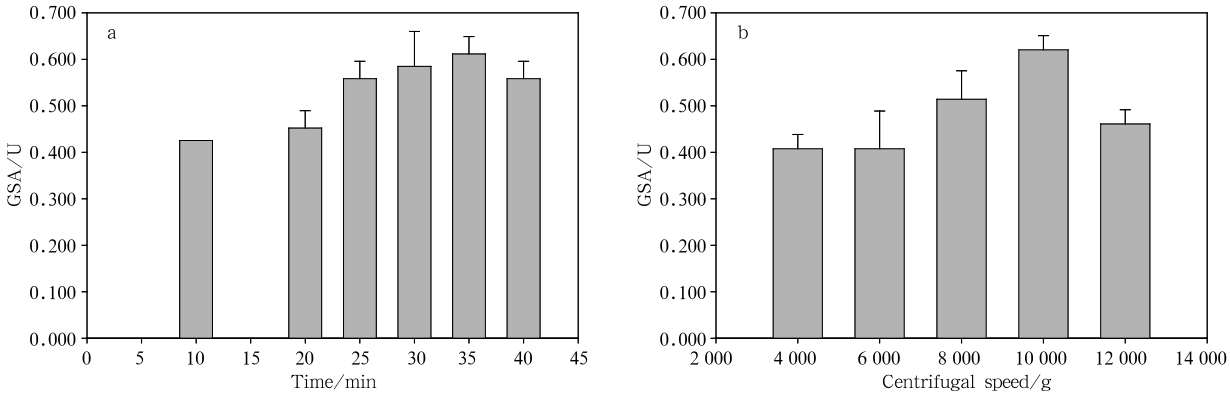

Fig.4. Optimization of centrifugal times and speeds for extraction of GS by the HC method. Activities are expressed as a mean value (n=3), and an error bar ($\pm SE$) is provided for each column. a. Centrifugal time; b. centrifugal speed.

4 Discussion

GS is an important enzyme in marine phytoplankton and has received intensive study since Tempest et al. (1970) discovered the occurence of the enzyme GOGAT and the importance of an alternate route of ammonium assimilation via mediatioin of the enzymes GS coupled with GOGAT. However, little work has been done on in situ phytoplankton samples due to a lack of standard enzyme preparation method. Previous studies used various enzyme extraction methods for GS analysis (Ei Alaoui et al., 2003, 2001; García-Fernández et al., 1997; Robertson and Alberte, 1996; Humanes et al., 1995; García-Fernández et al., 1994; Bressler and Ahmed, 1984), but the centrifugal speed and time varied greatly, which resulted in uncertainty and incompatibility of these studies.

Our study compared three crude enzyme preparation methods and optimized the crude enzyme preparation method. The results showed that HC was the most efficient method to prepare GS crude enzyme for diatom species, while UF was the best method to extract GS from dinoflagellate species, whereas UC method was not a good choice for preparation of GS crude enzyme. Many previous studies usually used high centrifugal speed (more than 20 000 g) or ultracentrifugation to prepare crude GS enzyme (García-Fernández et al., 1997; Robertson and Alberte, 1996; Humanes et al., 1995; García-Fernández et al., 1994; Bressler and Ahmed, 1984), but the reason for this is not made clear although high centrifugal speed might have removed those impurities which existed in the crude enzymes and minimized the interactions between impurities and chemical reagents. However, the present study showed no difference between the time zero control values of the two methods, and furthermore, GS activities declined significantly in all samples prepared with ultracentrifugation, indicating that ultracentrifugation caused mass loss of GS in the crude enzyme extract. The results of GS biosynthetic activity also indicated that GS activity in the HC method was much higher than that in the UC method. Ultrafiltration has been widely used to purify enzymes and proteins in various organisms and has proved to be an efficient method. This study tried using it to prepare crude enzyme extracts and good results were obtained for two dinoflagellate species, but it was not ideal for diatom species, which reflected the species specificity of GS.

At present, three isoforms of GS, GS I, GS II and GS III have been characterized based on the molecular size and submit number of the homoenzyme (Robertson and Alberte, 1996; García-Fernández et al., 1994; Reyes and Florencio, 1994). For most eukaryotic phytoplankton, GS II is the main isoform of the three GS isoforms (Robertson and Alberte, 1996; Forde and Cullimore, 1989). However, the composition of GS is complicated in phytoplankton under different conditions. This study indicated that the GS compositions in diatom and dinoflagellate species might be different, thus resulting in different GS extraction efficiency using the HC and UF methods. The results from field samples also demonstrated this point. GSA of field samples collected from Xiamen Harbor presented

the same characteristic as diatom species, indicating that the composition of this phytoplankton sample was dominated by diatom species. Results from the microscopic examination proved this, the phytoplankton population of the field sample proved to be composed mainly of diatoms, *Skeletonema costatum* for example. Thus, in the analysis of laboratory and in situ GSA, we should choose a suitable GS crude enzyme preparation method based on the phytoplankton species and phytoplankton composition of the field samples.

In summary, this study compared three crude enzyme preparation methods for GSA analysis, and optimized a crude enzyme preparation method. The results demonstrated that the HC method was best for the preparation of crude enzyme for GS analysis in diatom species and diatom-dominant samples, while the UF method was best for the extraction of GS from dinoflagellate species and dinoflagellate-dominant samples. In the case of the HC method, the optimal centrifugal speed and time were 10 000 g and 35 min respectively. Furthermore, this method was easy and practical to operate on the board for in situ GSA studies at sea.

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Glutamine synthetase (GS) has been purified from the cytosolic fraction of non-frozen human brain tissue. The purified GS migrated as a main band around 44 kD on reducing SDS-PAGE. Two-dimensional electrophoresis revealed heterogeneity within within subunits of GS. The masses of eight different peptides from a tryptic digest of GS as measured by high resolution MALDI-MS matched with the respective masses from an in silico tryptic fingerprint of the Swiss-Prot datase entry of human liver GS, proving that at least 24% of the primary sequences of GS from brain and liver are identical. Sedimentation equilibrium profiles obtained from analytical ultracentrifugation experiments at 10 deg C showed that human brain GS is mainly octameric. The quaternary structure of human brain GS at 10 muM (subunit concentration) was not significantly affected by cations, such as magnesium (5 and 20 mM) or manganese (0.2 and 1 mM) within the range of pH 7.1-7.8.

2. 外文期刊 Hu B. Petrasch-Parwez E. Laue MM. Kilimann MW Molecular characterization and immunohistochemical localization of palmdelphin, a cytosolic isoform of the paralemmin protein family implicated in membrane dynamics

Palmdelphin is a newly identified cytosolic isoform of paralemmin-1, a lipid raft-associated protein implicated in cell shape control. Like paralemmin-1, palmdelphin is phosphorylated, giving rise to electrophoretic band heterogeneity that is most pronounced in the brain. In ultracentrifugation and gel filtration palmdelphin behaves as a non-globular monomer. Its C-terminal region binds glutamine synthetase. Immunohistochemical analysis of the rat brain shows a prominent localization of palmdelphin in the cerebral cortex, hippocampus, amygdala, septum, indusium griseum, piriform cortex, nucleus supraopticus, and nucleus of the lateral olfactory tract. Many of the circumscript palmdelphin-positive areas are related to the olfactory system. Immunoperoxidase electron microscopy reveals a discontinuous distribution of palmdelphin immunoreactivity, in the form of spots scattered throughout the cytoplasm of selected neuronal perikarya and dendrites, including dendritic spines, often in association with endomembranes, and in a pattern which is similar to that of the cytoplasmic fraction of paralemmin-1. In subcellular fractionation experiments palmdelphin behaves as a cytosolic protein which, however, can be partially recruited from cytosol to the detergent-resistant fraction of a membrane/cytoskeletal cell ghost preparation. These observations suggest that palmdelphin may peripherally associate with endomembranes or cytoskeleton-linked structures.

3. OA论文 Donohue. T J. Bernlohr. R W Properties of the Bacillus licheniformis A5 glutamine synthetase purified from cells grown in the presence of ammonia or nitrate.

The glutamine synthetase from Bacillus licheniformis A5 was purified by using a combination of polyethylene glycol precipitation and chromatography on Bio-Gel A 1.5m. The resulting preparation was judged to be homogeneous by the criteria of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, equilibrium analytical ultracentrifugation, and electron microscopic analysis. The enzyme is a dodecamer with a molecular weight of approximately 616,000, and its subunit molecular weight is 51,000. Under optimal assay conditions (pH 6.6, 37 degrees C) apparent Km values for glutamate, ammonia, and manganese.adenosine 5'-triphosphate (1:1 ratio) were 3.6, 0.4, and 0.9 mM, respectively. Glutamine synthetase activity was inhibited approximately 50% by the addition of 5 mM glutamine, alanine, glycine, serine, alpha-ketoglutarate, carbamyl phosphate, adenosine 5'-diphosphate, or inosine 5'-triphosphate to the standard glutamine synthetase assay system, whereas 5 mM adenosine 5'-monophosphate or pyrophosphate caused approximately 90% inhibition of enzyme activity. Phosphorylribosyl pyrophosphate at 5 mM enhanced activity approximately 60%. We were unable to detect any physical or kinetic differences in the properties of the enzyme when it was purified from cells grown in the presence of ammonia or nitrate as sole nitrogen source. The data indicate that B. licheniformis A5 contains one species of glutamine synthetase whose catalytic activity is not regulated by a covalent modification system.